PATENT

Attorney Docket No.: 040853-01-5108-US
Client Ref. No.: NEO00073

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation Number: 1113
Robert Bayer	Examiner: Rao, Manjunath
Application No.: 09/855,320	Technology Center/Art Unit: 1652
Filed: May 14, 2001	DECLARATION OF DR. ROBERT BAYER UNDER 37 C.F.R. § 1.132
For: IN VITRO MODIFICATION OF GLYCOSYLATION PATTERNS OF RECOMBINANT GLYCOPEPTIDES	"THOMAS DECLARATION"
Customer No.: 43850	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert Bayer, Ph.D. declare as follows:

1. I am Senior Director of Research at Neose Technologies, Inc. My duties include directing the research operations of approximately 20 scientists at Neose's San Diego facility. Among these research operations are Neose's GlycoAdvance collaborations. GlycoAdvance is the name of our *in vitro* glycosylation technologies. I have over 14 years experience in this technology area. My *Curriculum Vitae* is attached as Exhibit 2A.

2. I am an inventor of the subject matter claimed in U.S. Patent Application No. 09/855,320 entitled "*In Vitro* Modification of Glycosylation Patterns of Recombinant Glycopeptides" ("the '320 Application"). I am familiar with the material contained in this application.

3. The '320 Application discloses glycopeptides with a "substantially uniform glycosylation pattern" prepared through contacting a glycopeptide having a glycosyl acceptor with a glycosyltransferase and a glycosyl donor moiety. The '320 Application further discloses and also claims a glycopeptide with a "substantially uniform fucosylation pattern" prepared by contacting a glycopeptide having a fucosyl acceptor with a fucosyltransferase and a fucosyl donor moiety.

4. In an earlier filed Declaration by Dr. David Zopf ("Zopf Declaration"), a scientific paper, Thomas, L.J. *et al.*, *Glycobiology* **14(10)**: 883-893 (2004) ("Thomas"), was presented. Thomas was the result of a collaboration between the assignee of the '320 Application and Avant Immunotherapeutics, and is attached as Exhibit 2B.

5. I am submitting this declaration to clarify the substantial identity between the methods and results of the '320 Application and those of the Thomas reference.

6. The starting material in an example of the '320 Application and in Thomas were substantially identical. See paragraph 13.

7. The starting material in an example of the '320 Application and in Thomas were submitted to substantially identical fucosylation conditions. The only difference between the examples disclosed in the two documents is the ratio of fucosyl donor to fucosyl acceptor substrate. In the '320 Application, this ratio is 14:1 (donor:acceptor); in Thomas, this ratio is 7:1 (donor:acceptor). See paragraph 14.

8. Because substantially the same starting materials were submitted to substantially the same fucosylation conditions, one of skill in the art would appreciate that the products of the method of the '320 Application and those of Thomas are substantially the same.

9. The '320 Application claims a composition comprising a glycopeptide having a "substantially uniform fucosylation pattern."

10. The term "substantially uniform glycosylation pattern" is defined on page 15, lines 1-3 and 12-15 of the '320 Application as follows:

> A "substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). . .
>
> The term "substantially" in the above definitions of "substantially uniform" generally means at least about 60%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

According to this definition, a minimum of 60% of the glycosyl acceptor moieties on a glycopeptide must be glycosylated in order for the glycopeptide to possess a "substantially uniform glycosylation pattern". Therefore, for the specific case of fucose, a minimum of 60% of the fucosyl acceptor moieties on a glycopeptide must be fucosylated in order for the glycopeptide to possess a "substantially uniform fucosylation pattern".

11. The 7:1 fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern". Therefore, the products subjected to the 14:1 fucosylation conditions of the '320 Application also possess a "substantially uniform fucosylation pattern". See paragraphs 16-25.

12. To the extent that the fucosylation conditions differ, the fucosylation conditions of the '320 Application would not be expected to reduce the total percentage of fucosylation, because there are more fucose donors present in the reaction mixture.

The starting materials in the '320 Application and in Thomas were substantially identical.

13. In both Thomas and the '320 Application, the starting material was sCR1-S. In both Thomas and the '320 Application, this starting material was produced *in situ* from a sialylation reaction. The similarity in the starting materials was revealed through fluorophore-assisted carbohydrate electrophoresis gel ("FACE gel") analysis. The FACE gel analysis of the

starting material for Thomas was disclosed in lane 3 of Figure 1. The FACE gel analysis of the starting material for the '320 Application was disclosed in the 'sialylated' lane of Figure 3. The band patterns in both lane 3 and the 'sialylated' lane were the same. The higher of the two bands was a monosialylated glycan product (DP = 7) and the lower of the two bands was a disialylated glycan product (DP = 6.2). The structures of these glycan products are attached as part of Exhibit 2C. The monosialylated glycan product is structure A in Exhibit 2C while the disialylated glycan product is structure B of Exhibit 2C.

The same starting materials were subjected to substantially identical fucosylation conditions in the methods of Thomas and in the methods of the '320 Application.

14. Substantially identical fucosylation conditions are disclosed in Thomas and the '320 Application. These conditions are attached as Exhibit 2D. Thomas and the '320 Application disclose that the reaction temperatures as well as concentrations of fucose acceptors (sCR1-S), fucose donors (GDP-fucose) and fucosyltransferases (FT-VI) are the same. In addition, the ratio of fucosyltransferase to fucose acceptor (FT-VI : sCR1-S) is the same for Thomas (0.02 U FT-VI/mg sCR1-S) as for the '320 Application (0.02 U FT-VI/mg sCR1-S). The main difference between the two reaction conditions lies in the ratio of fucose donor to fucose acceptor. For Thomas, this ratio is 7:1. For the '320 Application, this ratio is 14:1. In other words, the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, if the 7:1 fucose donor : fucose acceptor fucosylation conditions of Thomas produce a "substantially uniform fucosylation pattern", then the 14:1 fucose donor : fucose acceptor fucosylation conditions of the '320 Application will also produce a "substantially uniform fucosylation pattern".

The products in the '320 Application and in Thomas are substantially identical.

15. The similarity in the products in Thomas and the '320 Application were revealed through FACE gel analysis. The FACE gel analysis of the products in Thomas was disclosed in lane 4 of Figure 1. The FACE gel analysis of the products in the '320 Application was disclosed

in the 'sialylated and fucosylated' lane of Figure 3. In both lane 4 and the 'sialylated and fucosylated' lane, a band was not present at DP 6.2, indicating the consumption of the unfucosylated, disialylated starting material. In both lane 4 and the 'sialylated and fucosylated' lane, one band was visible slightly below DP 7, with trace bands at higher DP values. The band contained a difucosylated, disialylated product. This product is structure D in Exhibit 2C.

The 7:1 donor : acceptor fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern".

16. The product of the 7:1 donor : acceptor fucosylation conditions in Thomas was subjected to HPLC and MALDI-TOF-MS analysis (Thomas, p. 884, column 1). The HPLC results are presented in paragraph 18. The MALDI-TOF-MS results are used in paragraphs 18-25 to determine whether the glycopeptide products of Thomas possess a "substantially uniform fucosylation pattern."

17. The HPLC analysis yielded monosaccharide content information, which was reported in the "sCR1-S/F" column in Table I of Thomas. According to structure D of Exhibit 2C, one of skill would expect the ratios of glucosamine: galactose: mannose: fucose: sialic acid to be 4: 2: 3: 3: 2. The reported relative amounts of these monosaccharides are 48: 27: 35: 39: 28, which reduces to 4: 2.3: 3: 3.3: 2.3. These experimental values correlate well with expected values.

18. The MALDI-TOF-MS analysis yielded molecular weight information about the fucosylation reaction products, which was reported in Figure 7C of Thomas. This molecular weight information was then converted into product percentages which are reported in Table III of Thomas.

19. Whether a glycopeptide has a "substantially uniform fucosylation pattern" was determined by dividing the total number of fucosylated acceptor sites by the total number of potential fucose acceptor sites. Paragraphs 20-23 detail the percentage of glycans that have one, two, three, or four acceptor sites, and the percentage of the acceptor sites that are fucosylated.

These percentages are then used in paragraph 24 to determine the total percentage of fucosylation, or whether a glycopeptide has a "substantially uniform fucosylation pattern".

20. Calculations for glycans with one acceptor site. The percentage of one-acceptor glycans having no fucose is 0.43 + 0.7 + 3.47 = 4.60. The percentage of one-acceptor glycans having one fucose is 0.95 + 1.15 + 7.07 = 9.17. The total percentage of glycans with one-acceptor glycans is (4.60 + 9.17) = 13.77. Of this number, 9.17/13.77 = 67% are fucosylated.

21. Calculations for glycans with two acceptor sites. The percentage of two-acceptor glycans having no fucose is 1.06. The percentage of two-acceptor glycans having one fucose is 1.28 + 2.93 + 17.26 + 0.13 = 21.60. The percentage of two-acceptor glycans having two fucoses is 0.58 + 6.12 + 51.24 + 0.82 = 58.76. The total percentage of glycans with two-acceptor glycans is (1.06 + 21.60 + 58.76) = 81.42. Of this number, 85.4% are fucosylated, as shown below.

$$\frac{(1.06)\times 0+(1.28+2.93+17.26+0.13)\times 1+(0.58+6.12+51.24+0.82)\times 2}{(81.42\times 2)}=85.4\%$$

22. Calculations for glycans with three acceptor sites. The percentage of three-acceptor glycans having no fucose is 0.16. The percentage of three-acceptor glycans having one fucose is 0.38. The percentage of three-acceptor glycans having two fucoses is 1.51 + 0.75 = 2.26. The percentage of three-acceptor glycans having three fucoses is 0.76. The total percentage of glycans with three-acceptor glycans is 3.55. Of this number, 67% are fucosylated, as shown below.

$$\frac{(0.16)\times 0+(0.38)\times 1+(1.51+0.75)\times 2+(0.75)\times 3}{(3.55\times 3)}=67\%$$

23. Calculations for glycans with four acceptor sites. The percentage of four-acceptor glycans having no fucose is 0.18. The percentage of four-acceptor glycans having one fucose is 0.35. The percentage of four-acceptor glycans having two fucoses is 0. The percentage of four-

acceptor glycans having three fucoses is 0. The total percentage of glycans with four-acceptor glycans is 0. The total percentage of glycans with four-acceptor glycans is 0.53. Of this number, 16% are fucosylated, as shown below.

$$\frac{(0.18)\times 0+(0.35)\times 1+(0)\times 2+(0)\times 3+(0)\times 4}{(0.53\times 4)}=16\%$$

24. Based on the percentages of paragraphs 20-23, the total percentage of fucosylation by Thomas is 83%, as shown below.

$$\frac{(13.77)\times 0.67+(81.42)\times 0.854+(3.55)\times 0.67+(0.53)\times 0.16}{(13.77+81.42+3.55+0.53)}=83\%$$

Since the total percentage of fucosylation is at least 60%, the glycopeptides produced by the methods of Thomas possess a "substantially uniform fucosylation pattern."

25. As mentioned in paragraphs 7 and 14, the fucosylation conditions between Thomas and the '320 Application are the same except that the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, since the 7:1 donor : acceptor fucosylation conditions of Thomas yield a glycopeptide with a "substantially uniform fucosylation pattern," the 14:1 donor : acceptor fucosylation conditions of the '320 Application also yield a glycopeptide with a "substantially uniform fucosylation pattern".

26. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: June 3 2005

Robert Bayer, Ph.D.

1-SF/7233111.1

Exhibit 2

PATENT

Attorney Docket No.: 040853-01-5108-US
Client Ref. No.: NEO00073

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Robert Bayer Application No.: 09/855,320 Filed: May 14, 2001 For: IN VITRO MODIFICATION OF GLYCOSYLATION PATTERNS OF RECOMBINANT GLYCOPEPTIDES Customer No.: 43850	Confirmation Number: 1113 Examiner: Rao, Manjunath Technology Center/Art Unit: 1652 DECLARATION OF DR. ROBERT BAYER UNDER 37 C.F.R. § 1.132 "THOMAS DECLARATION"

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert Bayer, Ph.D. declare as follows:

1. I am Senior Director of Research at Neose Technologies, Inc. My duties include directing the research operations of approximately 20 scientists at Neose's San Diego facility. Among these research operations are Neose's GlycoAdvance collaborations. GlycoAdvance is the name of our *in vitro* glycosylation technologies. I have over 14 years experience in this technology area. My *Curriculum Vitae* is attached as Exhibit 2A.

2. I am an inventor of the subject matter claimed in U.S. Patent Application No. 09/855,320 entitled "*In Vitro* Modification of Glycosylation Patterns of Recombinant Glycopeptides" ("the '320 Application"). I am familiar with the material contained in this application.

3. The '320 Application discloses glycopeptides with a "substantially uniform glycosylation pattern" prepared through contacting a glycopeptide having a glycosyl acceptor with a glycosyltransferase and a glycosyl donor moiety. The '320 Application further discloses and also claims a glycopeptide with a "substantially uniform fucosylation pattern" prepared by contacting a glycopeptide having a fucosyl acceptor with a fucosyltransferase and a fucosyl donor moiety.

4. In an earlier filed Declaration by Dr. David Zopf ("Zopf Declaration"), a scientific paper, Thomas, L.J. *et al.*, *Glycobiology* **14(10)**: 883-893 (2004) ("Thomas"), was presented. Thomas was the result of a collaboration between the assignee of the '320 Application and Avant Immunotherapeutics, and is attached as Exhibit 2B.

5. I am submitting this declaration to clarify the substantial identity between the methods and results of the '320 Application and those of the Thomas reference.

6. The starting material in an example of the '320 Application and in Thomas were substantially identical. See paragraph 13.

7. The starting material in an example of the '320 Application and in Thomas were submitted to substantially identical fucosylation conditions. The only difference between the examples disclosed in the two documents is the ratio of fucosyl donor to fucosyl acceptor substrate. In the '320 Application, this ratio is 14:1 (donor:acceptor); in Thomas, this ratio is 7:1 (donor:acceptor). See paragraph 14.

8. Because substantially the same starting materials were submitted to substantially the same fucosylation conditions, one of skill in the art would appreciate that the products of the method of the '320 Application and those of Thomas are substantially the same.

9. The '320 Application claims a composition comprising a glycopeptide having a "substantially uniform fucosylation pattern."

10. The term "substantially uniform glycosylation pattern" is defined on page 15, lines 1-3 and 12-15 of the '320 Application as follows:

> A "substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). . .
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> The term "substantially" in the above definitions of "substantially uniform" generally means at least about 60%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

According to this definition, a minimum of 60% of the glycosyl acceptor moieties on a glycopeptide must be glycosylated in order for the glycopeptide to possess a "substantially uniform glycosylation pattern". Therefore, for the specific case of fucose, a minimum of 60% of the fucosyl acceptor moieties on a glycopeptide must be fucosylated in order for the glycopeptide to possess a "substantially uniform fucosylation pattern".

11. The 7:1 fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern". Therefore, the products subjected to the 14:1 fucosylation conditions of the '320 Application also possess a "substantially uniform fucosylation pattern". See paragraphs 16-25.

12. To the extent that the fucosylation conditions differ, the fucosylation conditions of the '320 Application would not be expected to reduce the total percentage of fucosylation, because there are more fucose donors present in the reaction mixture.

The starting materials in the '320 Application and in Thomas were substantially identical.

13. In both Thomas and the '320 Application, the starting material was sCR1-S. In both Thomas and the '320 Application, this starting material was produced *in situ* from a sialylation reaction. The similarity in the starting materials was revealed through fluorophore-assisted carbohydrate electrophoresis gel ("FACE gel") analysis. The FACE gel analysis of the

starting material for Thomas was disclosed in lane 3 of Figure 1. The FACE gel analysis of the starting material for the '320 Application was disclosed in the 'sialylated' lane of Figure 3. The band patterns in both lane 3 and the 'sialylated' lane were the same. The higher of the two bands was a monosialylated glycan product (DP = 7) and the lower of the two bands was a disialylated glycan product (DP = 6.2). The structures of these glycan products are attached as part of Exhibit 2C. The monosialylated glycan product is structure A in Exhibit 2C while the disialylated glycan product is structure B of Exhibit 2C.

The same starting materials were subjected to substantially identical fucosylation conditions in the methods of Thomas and in the methods of the '320 Application.

14. Substantially identical fucosylation conditions are disclosed in Thomas and the '320 Application. These conditions are attached as Exhibit 2D. Thomas and the '320 Application disclose that the reaction temperatures as well as concentrations of fucose acceptors (sCR1-S), fucose donors (GDP-fucose) and fucosyltransferases (FT-VI) are the same. In addition, the ratio of fucosyltransferase to fucose acceptor (FT-VI : sCR1-S) is the same for Thomas (0.02 U FT-VI/mg sCR1-S) as for the '320 Application (0.02 U FT-VI/mg sCR1-S). The main difference between the two reaction conditions lies in the ratio of fucose donor to fucose acceptor. For Thomas, this ratio is 7:1. For the '320 Application, this ratio is 14:1. In other words, the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, if the 7:1 fucose donor : fucose acceptor fucosylation conditions of Thomas produce a "substantially uniform fucosylation pattern", then the 14:1 fucose donor : fucose acceptor fucosylation conditions of the '320 Application will also produce a "substantially uniform fucosylation pattern".

The products in the '320 Application and in Thomas are substantially identical.

15. The similarity in the products in Thomas and the '320 Application were revealed through FACE gel analysis. The FACE gel analysis of the products in Thomas was disclosed in lane 4 of Figure 1. The FACE gel analysis of the products in the '320 Application was disclosed

in the 'sialylated and fucosylated' lane of Figure 3. In both lane 4 and the 'sialylated and fucosylated' lane, a band was not present at DP 6.2, indicating the consumption of the unfucosylated, disialylated starting material. In both lane 4 and the 'sialylated and fucosylated' lane, one band was visible slightly below DP 7, with trace bands at higher DP values. The band contained a difucosylated, disialylated product. This product is structure D in Exhibit 2C.

The 7:1 donor : acceptor fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern".

16. The product of the 7:1 donor : acceptor fucosylation conditions in Thomas was subjected to HPLC and MALDI-TOF-MS analysis (Thomas, p. 884, column 1). The HPLC results are presented in paragraph 18. The MALDI-TOF-MS results are used in paragraphs 18-25 to determine whether the glycopeptide products of Thomas possess a "substantially uniform fucosylation pattern."

17. The HPLC analysis yielded monosaccharide content information, which was reported in the "sCR1-S/F" column in Table I of Thomas. According to structure D of Exhibit 2C, one of skill would expect the ratios of glucosamine: galactose: mannose: fucose: sialic acid to be 4: 2: 3: 3: 2. The reported relative amounts of these monosaccharides are 48: 27: 35: 39: 28, which reduces to 4: 2.3: 3: 3.3: 2.3. These experimental values correlate well with expected values.

18. The MALDI-TOF-MS analysis yielded molecular weight information about the fucosylation reaction products, which was reported in Figure 7C of Thomas. This molecular weight information was then converted into product percentages which are reported in Table III of Thomas.

19. Whether a glycopeptide has a "substantially uniform fucosylation pattern" was determined by dividing the total number of fucosylated acceptor sites by the total number of potential fucose acceptor sites. Paragraphs 20-23 detail the percentage of glycans that have one, two, three, or four acceptor sites, and the percentage of the acceptor sites that are fucosylated.

These percentages are then used in paragraph 24 to determine the total percentage of fucosylation, or whether a glycopeptide has a "substantially uniform fucosylation pattern".

20. Calculations for glycans with one acceptor site. The percentage of one-acceptor glycans having no fucose is 0.43 + 0.7 + 3.47 = 4.60. The percentage of one-acceptor glycans having one fucose is 0.95 + 1.15 + 7.07 = 9.17. The total percentage of glycans with one-acceptor glycans is (4.60 + 9.17) = 13.77. Of this number, 9.17/13.77 = 67% are fucosylated.

21. Calculations for glycans with two acceptor sites. The percentage of two-acceptor glycans having no fucose is 1.06. The percentage of two-acceptor glycans having one fucose is 1.28 + 2.93 + 17.26 + 0.13 = 21.60. The percentage of two-acceptor glycans having two fucoses is 0.58 + 6.12 + 51.24 + 0.82 = 58.76. The total percentage of glycans with two-acceptor glycans is (1.06 + 21.60 + 58.76) = 81.42. Of this number, 85.4% are fucosylated, as shown below.

$$\frac{(1.06)\times 0+(1.28+2.93+17.26+0.13)\times 1+(0.58+6.12+51.24+0.82)\times 2}{(81.42\times 2)}=85.4\%$$

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$$\frac{(0.16)\times 0+(0.38)\times 1+(1.51+0.75)\times 2+(0.75)\times 3}{(3.55\times 3)}=67\%$$

23. Calculations for glycans with four acceptor sites. The percentage of four-acceptor glycans having no fucose is 0.18. The percentage of four-acceptor glycans having one fucose is 0.35. The percentage of four-acceptor glycans having two fucoses is 0. The percentage of four-

acceptor glycans having three fucoses is 0. The total percentage of glycans with four-acceptor glycans is 0. The total percentage of glycans with four-acceptor glycans is 0.53. Of this number, 16% are fucosylated, as shown below.

$$\frac{(0.18)\times 0+(0.35)\times 1+(0)\times 2+(0)\times 3+(0)\times 4}{(0.53\times 4)}=16\%$$

24. Based on the percentages of paragraphs 20-23, the total percentage of fucosylation by Thomas is 83%, as shown below.

$$\frac{(13.77)\times 0.67+(81.42)\times 0.854+(3.55)\times 0.67+(0.53)\times 0.16}{(13.77+81.42+3.55+0.53)}=83\%$$

Since the total percentage of fucosylation is at least 60%, the glycopeptides produced by the methods of Thomas possess a "substantially uniform fucosylation pattern."

25. As mentioned in paragraphs 7 and 14, the fucosylation conditions between Thomas and the '320 Application are the same except that the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, since the 7:1 donor : acceptor fucosylation conditions of Thomas yield a glycopeptide with a "substantially uniform fucosylation pattern," the 14:1 donor : acceptor fucosylation conditions of the '320 Application also yield a glycopeptide with a "substantially uniform fucosylation pattern".

26. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: _____

_____
Robert Bayer, Ph.D.

1-SF/7233111.1

Exhibit 2A

Robert J. Bayer, Ph.D

6105 Dirac St.
San Diego, CA 92122

(858) 452-2313 (work)
(858) 453-8562 (home)

RESEARCH AND PROFESSIONAL EXPERIENCE

Neose Technologies, Inc., San Diego, CA **1999-Present**

Senior Director, Research

- Promoted to Senior Director, 2001.
- Recipient of Presidents award, 2002.
- Established West Coast facility of Neose (now 20 employees).
- Responsible for directing science at San Diego facility.
- Responsible for carrying out GlycoAdvance collaborations with partners.
- Responsible for monoclonal antibody glycosylation projects at Neose.
- Responsible for carrying out product optimization for three products in pre-clinical development.
- Inventor or co-inventor on over 15 pending patent applications.

Cytel, Inc., San Diego, CA **1990-1999**

Group Leader, Biochemistry

- Promoted to Senior Scientist in 1993, promoted to Group Leader in 1994.
- Recipient of President's Award in 1994.
- Responsible for molecular biology and biochemistry, including discovery research, cloning, expression, and purification methods development of glycosyltransferases used in manufacturing , and process development and generation of manufacturing directions for enzymatic carbohydrate synthesis.
- Discovered and patented method allowing near 100% yields for enzymatic carbohydrate synthesis, planned and carried out all scale-up from milligram to kilogram scale manufacturing for four products.
- Carried out or supervised the enzymatic steps under cGMP's in the manufacture of Phase I and Phase II clinical supplies of Cylexin.
- Developed or supervised the development of purification protocols for recombinant enzymes used at Cytel for Cylexin™ manufacturing from a variety of expression systems (mammalian, bacterial, fungal, insect cell).
- Developed methods for examining selectin-ligand interactions including ELISA, flow cytometry, fluorescence, and equilibrium dialysis.

As part of a collaborative effort with Abbott Laboratories, developed enzymatic synthesis technology to manufacture a human milk oligosaccharide for inclusion in their nutritional product lines.

- Successfully transferred technology to Abbott CAPD, and assisted in manufacturing multiple kilogram batches on site at Abbott used in human clinical trials.
- Developed enzymatic synthesis technology to produce other carbohydrates with business partners to be used in medical devices as well as consumer products.

- Developed glycoprotein remodeling technology to 'repair' incorrect glycosylation on recombinant glycoprotein therapeutics in collaboration with several other biotechnology companies.
- Developed or supervised development of assays suitable for high throughput screening for inhibitors of glycosyltransferases.
- Planned and carried out all purification process development, manufacturing, analytical development, and stability/formulation testing for CY1748, Cytel's humanized anti-P-selectin monoclonal antibody.

Pharmacia Genetic Engineering **1988-1990**
Research Scientist

Developed purification protocols for recombinant HIV proteins to be used in clinical diagnostics.
- Carried out or supervised microbial fermentation.
- Purification and characterization of recombinant proteins from procaryotic and eukaryotic expression systems; renaturation of proteins from inclusion bodies.
- Synthesis, purification, and conjugation of peptides for use as immunogens.

University of California at San Diego **1984-1988**
Postdoctoral Associate, Chemistry Department, Supervisor Prof. J. Kyte

- Developed a new methodology for determining the topology of membrane proteins, and applied this technology to the sodium-potassium ATPase.

Kyoto University **1983-1984**
Researcher, Department of Biophysics, Supervisor Prof. S.I. Ohnishi

- Investigated production of 2-D crystals of influenza virus hemagglutinin for structural determination.

Cornell University **1979-1983**
Graduate Student, Biochemistry and Mol. Biol., Supervisor Prof. G. Feigenson

- Developed a new reconstitution method for M13 coat protein. This was then used to characterize protein partitioning in model membranes undergoing divalent cation-induced phase changes using ESR and fluorescence spectroscopy.

University of Michigan **1976-1979**
Research Associate, Department of Biochemistry, Supervisor Prof. J. Shafer

- Used deuterium isotope effects, UV-visible difference spectroscopy, CD and NMR spectroscopy, and measurement of steady-state and pre-steady state kinetic parameters to elucidate the catalytic mechanism of D-serine dehydratase, an enzyme that uses pyridoxal phosphate as a cofactor.

Education, Awards, Honors, and Fellowships

Ph.D in Biochemistry, 1983 Cornell University
BS Chem with Distinction, High Honors in Chemistry, University of Michigan (1976)
Phi Lambda Upsilon, 1976
U of M Gomberg Prize in Chemistry, 1975, 1976
U of M Class Honors 1974-1976
U of M College Honors Program 1972-1976
National Merit Finalist, UDHS 1972

Publications

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Patents

Issued US Patents:

US Patent no. 6,033,667 Method for detecting the presence of P-selectin
Chesnut; Robert W.; Polley; Margaret J.; Paulson; James C.; Jones; S. Tarran; Saldanha; Jose W.; Bendig; Mary M.; Kriegler; Michael; Perez; Carl; **Bayer; Robert;** Nunn; Michael

US Patent No. 5,800,815 Antibodies to P-selectin and their uses
Chestnut; Robert W.; Polley; Margaret J.; Paulson; James C.; Jones; S. Tarran; Saldanha; Jose W.; Bendig; Mary M.; Kriegler; Michael; Perez; Carl ; **Bayer; Robert** ; Nunn; Michael

US Patent No. 5,922,577 Enzymatic synthesis of glycosidic linkages
Defrees; Shawn; **Bayer; Robert J.** ; Ratcliffe; Murray

US Patent No. 5,876,980 Enzymatic synthesis of oligosaccharides
DeFrees; Shawn; **Bayer; Robert J.**; Ratcliffe; Murray

US Patent No. 5,728,554 Improved Enzymatic Synthesis of Glycosidic Linkages
Bayer, R.J., DeFrees, S., & Ratcliffe, M.

US Patent No. 6,399,336 Practical in vitro sialylation of recombinant glycoproteins
Paulson; James C.; **Bayer; Robert J.**; Sjoberg; Eric

US Patent No. 6,030,815 Enzymatic synthesis of oligosaccharides
DeFrees; Shawn; **Bayer; Robert J.**; Ratcliffe; Murray

US Patent Applications

Currently inventor or co-inventor on over 15 pending US patent applications.

Exhibit 2B

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Production of a complement inhibitor possessing sialyl Lewis X moieties by *in vitro* glycosylation technology

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Recombinant soluble human complement receptor type 1 (sCR1) is a highly glycosylated glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. sCR1-sLe^x produced in the FT-VI-expressing mutant CHO cell line LEC11 exists as a heterogeneous mixture of glycoforms, a fraction of which include structures with one or more antennae terminated by the sialyl Lewis X (sLe^x) [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc] epitope. Such multivalent presentation of sLe^x was shown previously to effectively target sCR1 to activated endothelial cells expressing E-selectin. Here, we describe the use of the soluble, recombinant α2-3 sialyltransferase ST3Gal-III and the α1-3 fucosyltransferase FT-VI *in vitro* to introduce sLe^x moieties onto the N-glycan chains of sCR1 overexpressed in standard CHO cell lines. The product (sCR1-S/F) of these *in vitro* enzymatic glycan remodeling reactions performed at the 10-g scale has approximately 14 N-glycan chains per sCR1 molecule, comprised of biantennary (90%), triantennary (8.5%), and tetraantennary (1.5%) structures, nearly all of whose antennae terminate with sLe^x moieties. sCR1-S/F retained complement inhibitory activity and, in comparison with sCR1- sLe^x produced in the LEC11 cell line, contained twice the number of sLe^x moieties per mole glycoprotein, exhibited a twofold increase in area under the intravenous clearance curve in a rat pharmacokinetic model, and exhibited a 10-fold increase in affinity for E-selectin in an *in vitro* binding assay. These results demonstrate that *in vitro* glycosylation of the sCR1 drug product reduces heterogeneity of the glycan profile, improves pharmacokinetics, and enhances carbohydrate-mediated binding to E-selectin.

Key words: glycoengineering/glycoprotein remodeling/glycosylation/glycosyltransferase

Introduction

Soluble complement receptor type 1 (sCR1) is a recombinant glycoprotein that has been shown to inhibit the progression of the complement cascade in both the classical and alternative pathways by inhibiting the stable formation of C3 and C5 convertases and by serving as a cofactor in the proteolytic degradation of C3b and C4b by Factor I (Weisman *et al.*, 1990). The administration of sCR1 has been shown to be effective in a number of animal disease models of human complement-dependent ischemia-reperfusion injury for tissues, such as heart (Lazar *et al.*, 1999), liver (Lehmann *et al.*, 1998), hind limb (Kyriakides *et al.*, 2001a), lung (Naka *et al.*, 1997), and intestine (Willianis *et al.*, 1999). Complement inhibition by sCR1 has been shown to reduce hyperacute rejection (Pruitt *et al.*, 1997) and enhance graft survival in many established transplant models (Kallio *et al.*, 2000; Pratt *et al.*, 1996; Stamnberger *et al.*, 2000).

In some clinical situations, complement inhibition therapy could be more effective if it were targeted directly to sites of endothelial activation. At sites of inflammation, activated endothelial cells express E-selectin and P-selectin, surface adhesins with carbohydrate-binding domains that recognize the carbohydrate epitope, sLe^x (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) (Lasky, 1995).

Previously we have described sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), a variant of the sCR1 glycoprotein conveniently produced in LEC11 cells transfected with the sCR1 gene. LEC11 is a mutant Chinese hamster ovary (CHO) cell line that expresses fucosyltransferase VI (FT-VI), a Golgi enzyme capable of adding fucose in α1-3 linkage to GlcNAc in oligosaccharide chains that terminate with either Galβ1-4GlcNAcβ1 ... or Neu5Acα2-3Galβ1-4GlcNAcβ1 ...(Zhang *et al.*, 1999). Of the 25 potential N-glycosylation sites within the sCR1 polypeptide sequence, 13–15 are occupied, the majority with biantennary chains, creating the possibility for as many as 30 sLe^x moieties per molecule of sCR1-sLe^x. However, a previously reported analysis of the N-glycans of sCR1-sLe^x showed heterologous oligosaccharides with a variety of partially sialylated and fucosylated structures yielding less than the maximal number of sLe^x moieties (Picard *et al.*, 2000; Rittershaus *et al.*, 1999). Similar heterogeneity of glycans in CHO-expressed glycoproteins has been described previously and attributed to incomplete Golgi processing, post-secretion degradation due to glycohydrolases released into cell culture media, or both (Goochee *et al.*, 1991; Jenkins *et al.*, 1996).

In this article we describe a process to introduce sLe^x moieties onto the N-glycan chains of sCR1 produced in staudard CHO cell lines using *in vitro* enzymatic synthesis.

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This method employs serial treatment of sCR1 with soluble recombinant rat ST3Gal-III and human FT-VI to give an sCR1-sLe^x product, designated sCR1-S/F (for differentiation from the LEC11 product) in which the antennae of N-glycans are nearly uniformly terminated with sLe^x epitopes. The benefits of *in vitro* glycan remodeling include improved pharmacokinetics, enhanced binding to E-selectin, and a means to improve product homogeneity. Enzymatic remodeling is demonstrated at the 10-g scale.

Results

In vitro *remodeling of sCR1 glycans*

sCR1 (250 mg) expressed in CHO cells was sialyated by treatment with ST3Gal-III plus CMP-sialic acid to give a product designated sCR1-S. After an aliquot was removed from the reaction mixture for analysis, the remaining sCR1-S was fucosylated in the same reaction vessel by the addition of FT-VI plus GDP-fucose to give a product designated sCR1-S/F. After purification by serial chromatography on ceramic hydroxyapatite and Q Sepharose, the reaction products had the same retention time and percent purity (98.5%) by reversed phase high-pressure liquid chromatography (RP-HPLC) as the starting material, sCR1 (data not shown). Chemical and functional properties of these molecules were compared with those of sCR1-sLe^x, a molecule previously produced in the FT-VI-expressing LEC11 CHO cell line and shown to contain some N-linked biantennary glycans terminated with the sLe^x tetrasaccharide (Picard *et al.*, 2000).

From the mannose content of sCR1, sCR1-S, sCR1-S/F, and sCR1-sLe^x (Table I) it may be inferred that these molecules contain ~13–15 N-glycan chains per mol protein (assuming 3 mol mannose per N-glycan chain). The fluorophore-assisted carbohydrate electrophoresis (FACE) oligosaccharide profile for sCR1 (Figure 1) shows three major bands consistent with a biantennary structure containing zero, one, or two sialic acid residues, as described previously (Picard *et al.*, 2000). The monosaccharide composition of sCR1 (Table I) suggests that ~57% of total galactosyl residues are substituted with sialic acid (19 mol sialic acid/ 33.2 mol galactose). By comparison, the FACE oligosaccharide profile for sCR1-S (Figure 1) shows one major band that migrates at a position consistent with a biantennary structure containing two sialic acid residues, and monosaccharide analysis reveals the galactose/sialic acid ratio to be 1:1 (Table I).

FACE analysis of glycans from sCR1-S/F, prepared by enzymatic fucosylation of sCR-S, suggests that N-glycans are predominantly biantennary and that fucosylation at both antennae is nearly complete (Figure 1). The dominant oligosaccharide band derived from sCR1-S/F was cut out and extracted from the gel. Sequential removal of monosaccharide residues from the extracted glycoprotein using specific glycosidases gave products with mobilities consistent with α1-6 core-fucosylated, biantennary N-glycans (Figure 2). Monosaccharide analysis of sCR1-S/F shows the presence of 39.3 moles fucose per mol sCR1-S/F, a figure in agreement with the prediction from theory that 39–45 fucose residues per mol protein would be present if all N-glycans were core fucosylated and enzymatic fucosylation of antennary GlcNAc residues were complete.

The FACE oligosaccharide profile for sCR1-sLe^x, a glycoprotein produced in LEC11 CHO cells, shows at least seven bands (Figure 1) with some common to sCR1 and others shown previously (Picard *et al.*, 2000) to represent core fucosylated structures with α1-3 fucosylation at one or more antennae. Heterogeneity in the degree of fucosylation of the N-glycan chains from sCR1-sLe^x also can be appreciated from the results of monosaccharide analysis (Table I). For example, it may be calculated (assuming 3 mannose residues per chain) that sCR1-sLe^x contains an average of 2.5 fucosyl residues per glycan chain. By contrast, the fucose content per glycan chain increases from 0.95 for

Table I. Monosaccharide Content (mol/mol glycoprotein) by HPLC analysis

	sCR1	sCR1-S	sCR1-S/F	sCR1-sLe^x
Glucosamine	62	48	48	62
Galactose	33	28	27	38
Mannose	44	39	35	40
Fucose	16	12	39	33
Sialic acid	19	30	28	27
Sialic acid/galactose	0.57	1.09	1.06	0.70
Glycosylation sites/sCR1	15	13	12	13
Estimated sLe^x/sCR1-sLe^x*	n.a.	n.a.	28	14

*Estimated sLe^x/sCR1-sLe^x = (Fuc/sCR1-sLe^x ÷ sites/sCR1-sLe^x) × Sial/Gal ratio.

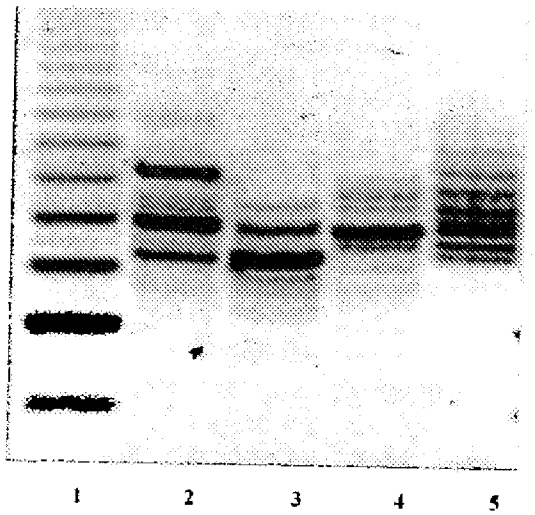


Fig. 1. FACE profiling of oligosaccharides from sCR1-sLe^x and sCR1 before and after enzymatic remodeling: (1) Glyko oligosaccharide standard ladder, (2) sCR1, (3) sCR1-S, (4) sCR1-S/F, (5) sCR1-sLe^x. The oligosaccharide profile of sCR1 (lane 2) contains predominantly bands representing biantennary structures with two sialic acids (bottom band), one sialic acid (middle band), and no sialic acids (top band).

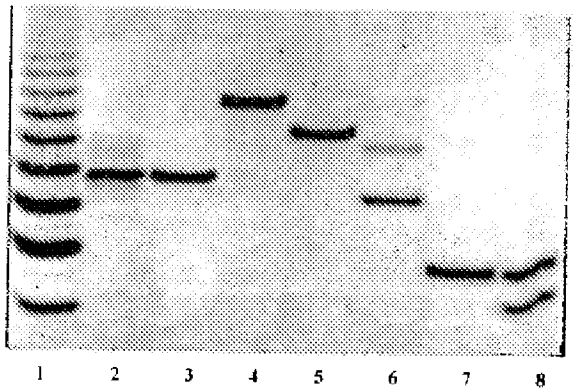


Fig. 2. FACE analysis of oligosaccharides from sCR1-S/F after serial treatment with glycosidases. The dominant oligosaccharide band derived from sCR1-S/F (lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was digested sequentially to remove each monosaccharide residue starting at the terminal sialic acid residue and ending at the trimannosyl core: (1) Glyko oligosaccharide standard ladder; (2) total N-linked oligosaccharides of sCR1-S/F; (3) purified dominant band (band 1) from lane 2; (4) band 1 treated with NANaseIII (cleaves α2-3, 4, 6, 8, and 9 linked sialic acid); (5) band 1 treated with NANaseIII and FucaseIII (cleaves α1-3 and 4 fucose); (6) band 1 treated with NANaseIII, FucaseIII, and GalaseIII (cleaves terminal galactose); (7) band 1 treated with NANaseIII and FucaseIII, GalaseIII, and hexosaminidase; (8) standard trimannosyl core N-glycans with (upper band) and without α1-6 fucose.

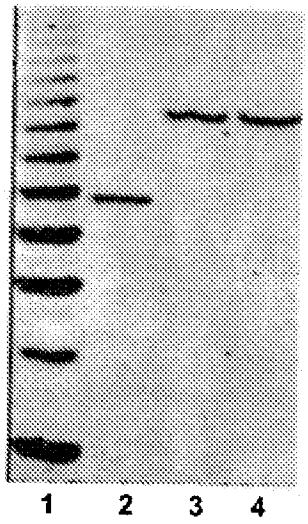


Fig. 3. FACE analysis of oligosaccharides from sCR1-S/F treated with sialidases. The dominant oligosaccharide band (band 1) derived from sCR1-S/F (see Figure 2, lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was subjected to enzymatic digestion to remove terminal sialic acid: (1) Glyko oligosaccharide standard ladder; (2) band 1 from sCR1-S/F; (3) band 1 treated with NANaseI (cleaves α2-3 linked sialic acid); (4) band 1 treated with NANaseIII (cleaves α-3, 4, 6, 8, and 9 linked sialic acid).

sCR1-S to 3.3 for sCR1-S/F, a result that correlates well with the single band visualized by FACE analysis of sCR1-S/F (Figure 1).

Oligosaccharide sequencing using FACE

The linkage of terminal sialic acids on sCR1-S/F was assessed by digestion with specific neuraminidases (Figure 3). Complete removal of sialic acid by treatment of band 1 from sCR1-S/F with NANase I indicates that sialic acid residues are α2-3 linked, as expected.

Optimization of sialylation reaction for scale-up

To establish conditions for scaleup of sialylation, sCR1 (5 mg/ml) was incubated with varying amounts of ST3Gal-III (10, 25, 75, 100, 200, 300 and 400 U/ml) and 5 mM CMP-sialic acid plus a trace amount of radiolabeled CMP-sialic acid for 24 h at 32°C. At an ST3Gal-III concentration of 150 mU/ml, incorporation of radiolabeled sialic acid reached 91% of maximum after 24 h and 100% at 48 h. The lowest concentration of enzyme required to give nearly maximum incorporation (~40 mol sialic acid/mol protein) under these conditions was 25 mU/ml ST3Gal-III (Figure 4). It should be noted that the contribution of triantennary and tetraantennary species may be responsible for the observation that more than 30 moles of sialic acid was added per mole of sCR1. Increasing the CMP-sialic acid concentration from 5 mM to 10 mM did not affect the level of sialylation of sCR1 at any of the ST3Gal-III concentrations tested (data not shown). HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of glycans

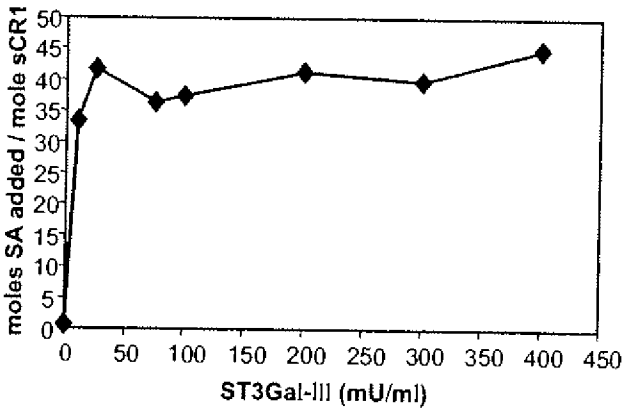


Fig. 4. Incorporation of sialic acid into sCR1 at increasing concentrations of ST3Gal-III in a 24-h reaction. The moles of sialic acid added are estimated from incorporation of radiolabeled CMP-sialic acid. Incorporated radiolabel is separated from free by gel filtration on a $TSKG2000_{SWXL}$ column.

released from sCR1-S revealed that at all concentrations of enzyme tested, the product contained predominantly disialylated, biantennary, core fucosylated N-glycans (data not shown). A concentration of 200 mU ST3Gal-III/ml was chosen for scale-up to ensure completeness of reaction.

Optimization of fucosylation

To establish conditions for scale-up of fucosylation, sCR1-S (5 mg/ml) was incubated with varying amounts of

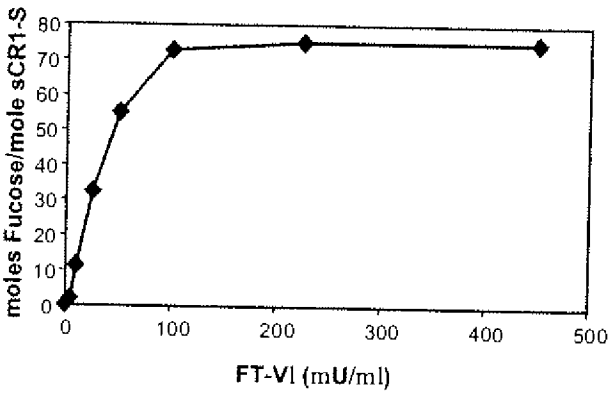


Fig. 5. Incorporation of fucose into sCR1-S at increasing concentrations of FT-VI in a 24-h reaction. The moles of fucose added are estimated from incorporation of radiolabeled GDP-fucose. Incorporated radiolabel is separated from free by gel filtration on a TSKG2000$_{SWXL}$ column.

FT-VI (10, 20, 40, 60, 100, 220, 440 mU/ml) and 5 mM GDP-fucose plus a trace of radiolabeled GDP-fucose for 24 h at 32°C. The lowest concentration of enzyme required to give nearly maximum incorporation of fucose under these conditions was 100 mU/ml FT-VI (Figure 5). Increasing the GDP-fucose concentration from 5 mM to 10 mM did not increase fucose incorporation at several different FT-VI concentrations tested (data not shown).

For products of reactions run at all concentrations of FT-VI $\geq$ 100 mU/ml, the glycan structures identified by HPLC and MALDI-TOF MS were almost the same and essentially indistinguishable from the structures described next for sCR1-S/F produced at the 10-g scale.

Remodeling at 10-g scale

Purified sCR1 (10 g in a volume of 2 L) was incubated first with ST3Gal-III plus CMP–sialic acid at 32°C for 36 h and then, following addition of FT-VI plus GDP-fucose, incubated at 32°C for another 36-h period..

FACE analyses of glycans from sCR1, sCR1-S, and sCR1-S/F for reactions performed at the 10-g scale (data not shown) were essentially indistinguishable from FACE results obtained at the 250-mg scale (Figure 1), suggesting that occupancy of potential acceptor sites for ST3Gal-III and FT-VI on sCR1 at the 10-g scale was nearly complete.

HPLC profiles for 2-AA-derivatized glycans of sCR1, sCR1-S, and sCR1-S/F are shown in Figure 6 and the percentages of glycan species estimated from integrated peak areas are summarized in Table II. After *in vitro* sialylation with ST3Gal-III, neutral glycans, comprising 50% of carbohydrate chains in sCR1, are reduced to 2% of chains in sCR1-S, and monosialo-glycans likewise decrease to from 35% in sCR1 to 17.5% in sCR1-S (Figure 6 and Table II). Overall, about 90% of N-glycans are biantennary and these chains contain an average of 1.8 sialic acid moieties per glycan. Among the minority of biantennary glycans on sCR1-S that are monosialylated, some lack galactose on one antenna, whereas others contain two galactosyl residues, only one of which is sialylated. The remaining 10% of

Table II. HPLC data summary of large scale remodeling

Glycan species	Native protein (sCR1) (%)	Sialylated protein (sCR1-S) (%)	Sialylated and fucosylated protein (sCR1-S/F) (%)
Neutral	50.5	2.0	4.0
1 charge	35.0	17.5	25.5
2 charges	13.0	70.5	68.5
3 charges	1.5	8.5	1.5
4 charges	ND*	1.5	0.5

*Not detected.

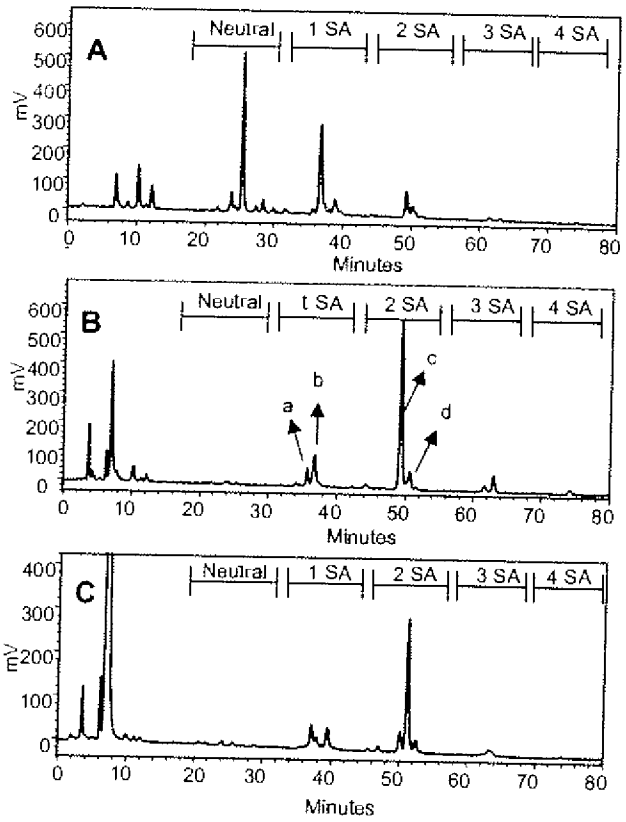
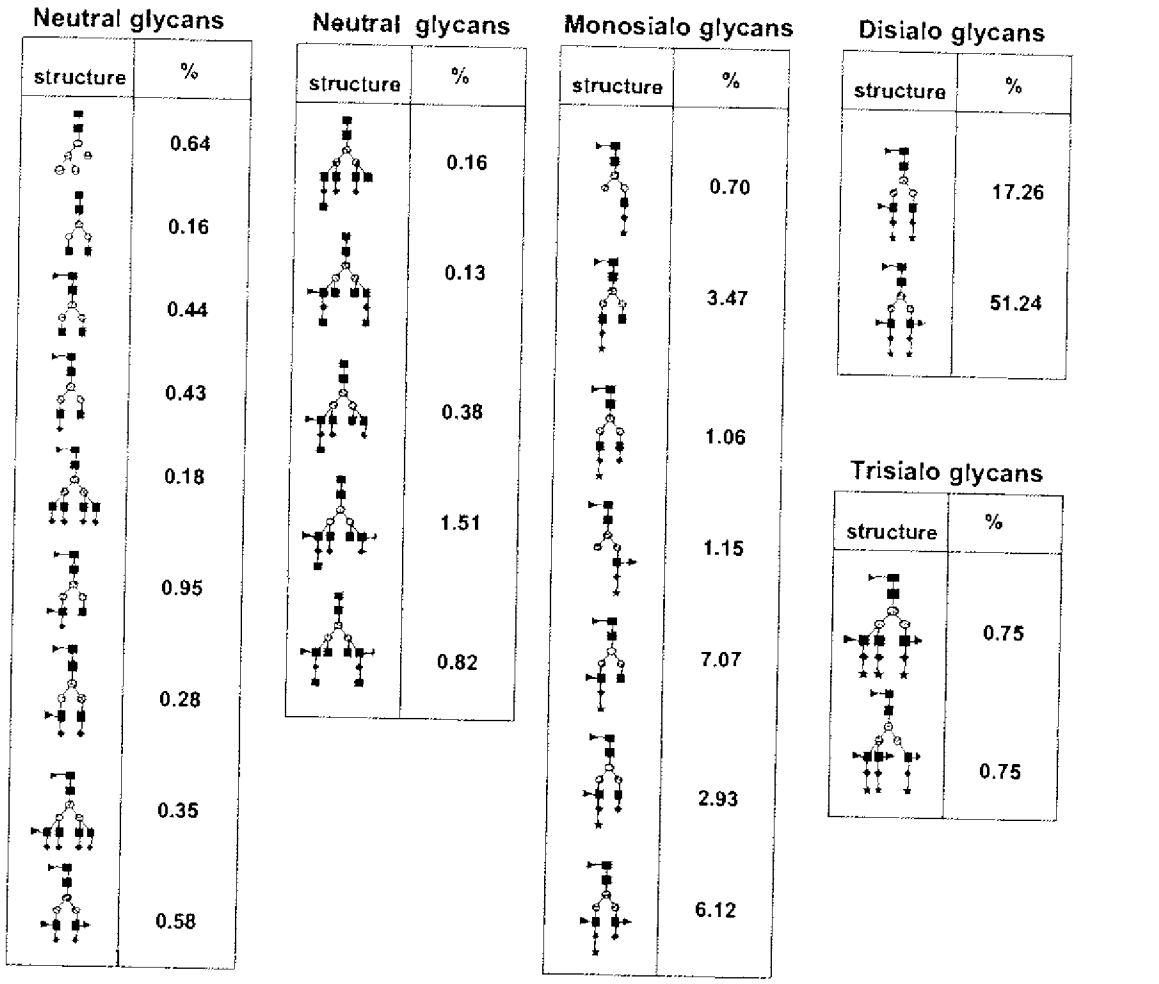


Fig. 6. RP-HPLC analysis of 2-AA-oligosaccharides before and after enzymatic remodeling at the 10-g scale: (A) sCR-1; (B) sCR1-S; (C) sCR1-S/F. MALDI-TOF MS analysis (data not shown) of 2-AA-oligosaccharides from sCR1-S (B) indicated that: peak a contains monosialylated biantennary glycans that lack terminal galactose on one antenna; peak b, constituting 12% of biantennary glycans, contains biantennary glycans with two galactose residues, but only one sialic acid; peaks c and d contain disialylated, biantennary glycans, with and without core fucose, respectively.

glycans are fully sialylated triantennary (8.5%) or tetra-antennary (1.5%) structures.

After fucosylation of sCR1-S to create sCR1-S/F, HPLC and MALDI-TOF MS analyses (Table III and Figure 7)

Table III. sCR1-S/F glycans



Blue squares represent N-acetylglucosamine, yellow circles represent mannose, green triangles represent fucose, red diamonds represent galactose, and asterisks represent sialic acid.

showed that more than 95% of the glycans were fucosylated by FT-VI. About 62% of the total N-glycans gained two fucose residues, and ~30% gained a single fucose residue. Failure to accept two fucosyl residues was in part due to missing galactosyl residues on one or more antennae. From these results it can be estimated that the sCR1-S/F molecules created by consecutive *in vitro* sialylation and fucosylation reactions contain, on average, 28 sLe^x epitopes per protein molecule, whereas sCR1-sLe^x, glycosylated and secreted by the FT-VI-expressing LEC11 CHO cell, contains ~14 sLe^x epitopes per protein molecule (Table 1).

To check the stability of sCR1 under conditions of incubation with glycosyltransferases, a small amount of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after each remodeling reaction. There was no evidence of degradation of the polypeptide after incubation with either ST3Gal-III or FT-VI (data not shown).

Pharmacokinetics

When sCR1-S prepared at the 250-mg scale was injected intravenously into rats, the observed area under the curve (AUC_{last}) was twofold greater than the AUC_{last} for sCR1 ($p < 0.004$), indicating a significantly greater exposure of the more completely sialylated form of the complement inhibitor to intravascular cells following dosing (Figure 8).

In vitro *antihemolytic activity*

The $1H_{50}$ values for sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F as inhibitors of human complement-mediated lysis of sheep red blood cells were found to be similar (Figure 9 and

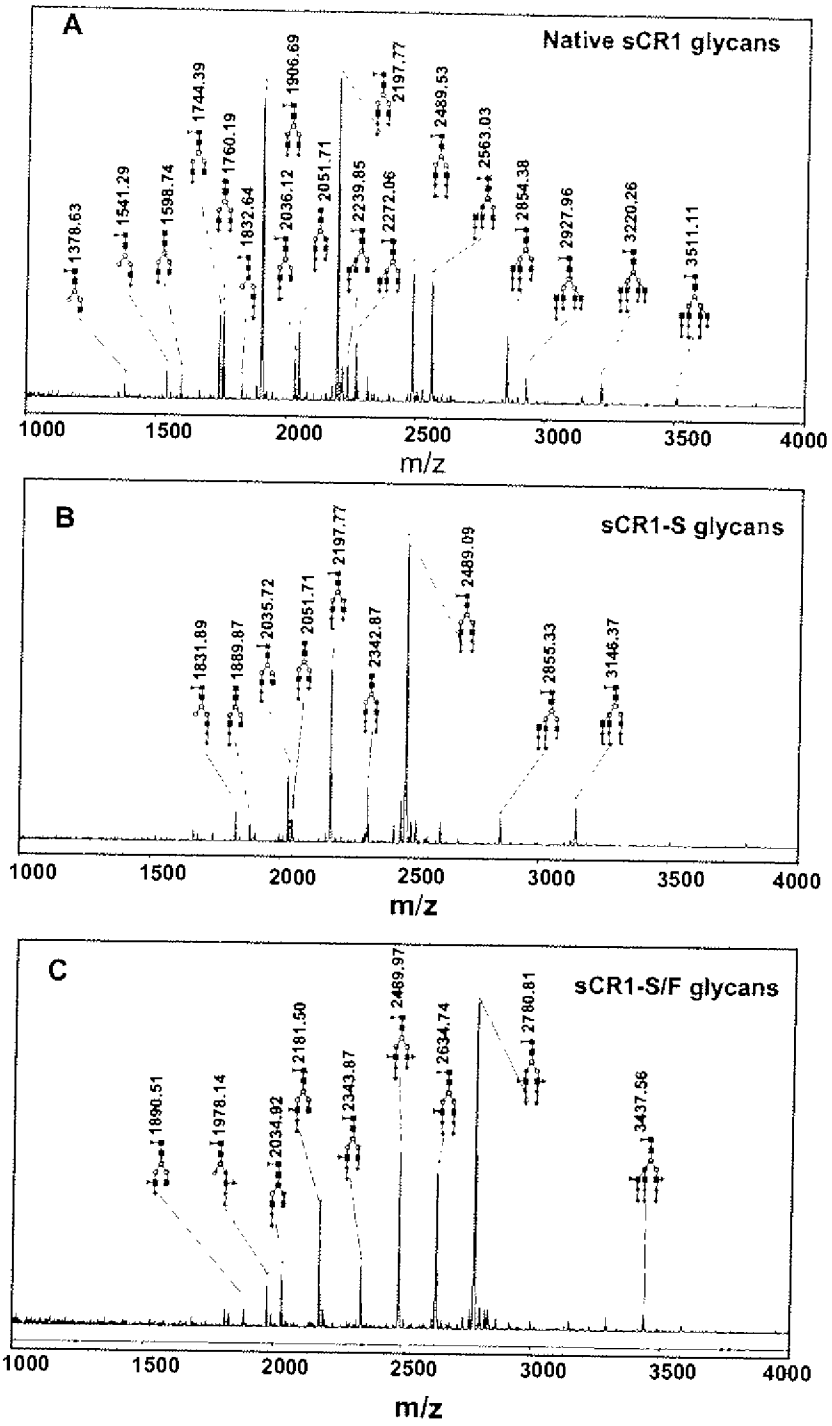


Fig. 7. MALDI-TOF analysis of total glycans from (A) sCR1, (B) sCR1-S, and (C) sCR1-S/F remodeled at the 10-g scale. The blue square is GlcNAc, the yellow filled circle is mannose, the green filled triangle is fucose, the red filled diamond is galactose, and the asterisk is sialic acid.

Table IV), indicating that *in vitro* glycosylation of sCR1 to yield sCR1-S or sCR1-S/F does not significantly impact the complement inhibitory properties of the molecule in the classical pathway.

In vitro *binding to E-selectin*

Figure 10 shows that sCR1-sLe^x and sCR1-S/F bind E-selectin in a concentration-dependent manner. The IC_{50}

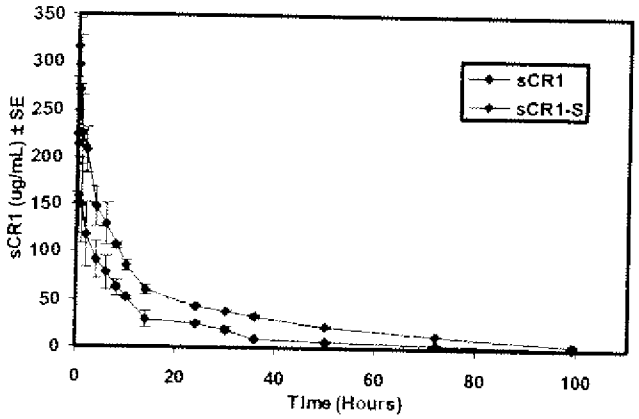


Fig. 8. The concentration of sCR1 and sCR1-S in plasma at various time points following bolus IV injection in rats.

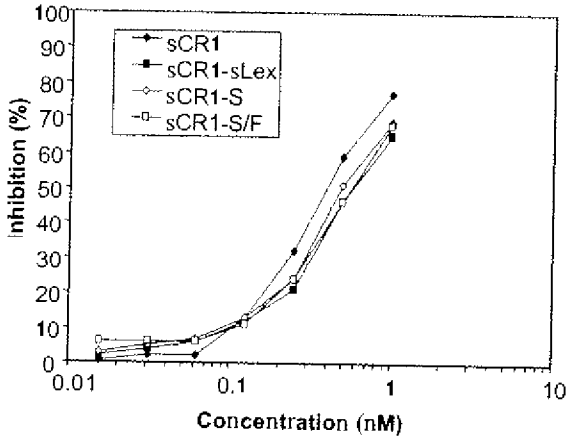


Fig. 9. Inhibition of red cell lysis via the classical pathway as a function of the concentration of sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F.

Table IV. Antihemolytic activity of modified sCR1 and sCR1-sLe^x

	IH_{50} (nM)
sCR1	0.41
sCR1-S	0.48 nM
sCR1-sLe^x	0.59
sCR1-S/F	0.59 nM

for sCR1-sLe^x from this plot is ~5 nM, and for sCR1-S/F ~0.4 nM. The observed 10-fold increase in inhibitory potency presumably is due to enhanced avidity, attributable to the increased density of sLe^x moieties on sCR1-S/F (28/mol) as compared with sCR1-sLe^x (14 per mol) (see Table I). The specificity of this binding was demonstrated by its calcium requirement and by the observation that sCR1

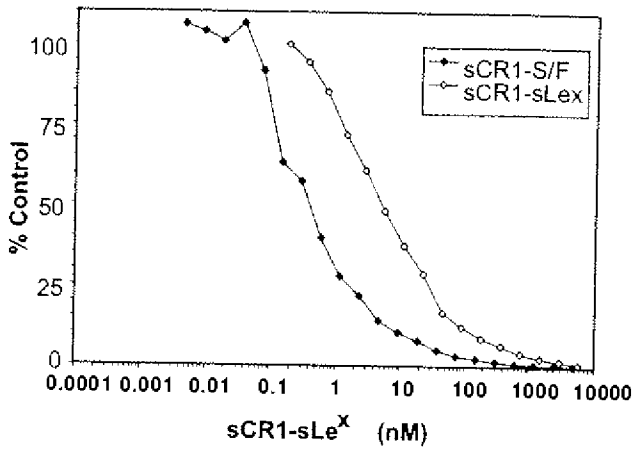


Fig. 10. Inhibition of PAA-sLe^x binding to E-selectin coated microtiter plates in the presence of varying concentrations of sCR1-sLe^x or sCR1-S/F.

(which does not contain any sLe^x structures) does not inhibit E-selectin binding at concentrations as high as 10 μM (data not shown).

Discussion

sCR1, made by standard CHO production methods, possesses predominantly biantennary oligosaccharides that are incompletely sialylated. We previously described an alternately glycosylated form of sCR1 called TP20 or sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), secreted by the FT-VI-expressing LEC11 CHO cell line and bearing sLe^x moieties on a fraction of its N-linked oligosaccharides. In this article we describe *in vitro* enzymatic remodeling of sCR1 by the stepwise application of two soluble recombinant glycosyltransferases in "one pot": The first step adds sialic acid to make sCR1-S, and the second adds fucose to make sCR1-S/F. The product of these glycan remodeling reactions contains an average of 28 sLe^x moieties per mol, as compared with 14 per mol found in CHO cell-produced sCR1-sLe^x.

That the sCR1 protein remains intact under conditions of glycan remodeling was demonstrated by RP-HPLC and SDS-PAGE analyses showing single polypeptides with expected molecular weights for sCR1-S and sCR1-S/F. Evidence for (1) conformational stability under conditions of the *in vitro* glycosylation reactions, and (2) preserved function despite variations in glycan structure, is provided by the observed near equivalence in bioactivity of sCR1, sCR1-S, sCR1-S/F, and CHO-produced sCR1-sLe^x in a standard complement inhibition assay.

The oligosaccharide structures associated with sCR1-S and sCR1-S/F were assessed by a number of methods. FACE profiling demonstrated a more fully sialylated set of glycoforms for sCR1-S as compared with sCR1 and nearly homogeneous, fully sialylated and fucosylated biantennary N-glycans for sCR1-S/F. Sequencing experiments using FACE provided supporting evidence that sialic acid

was linked α2-3 to galactose and that the predominant, single oligosaccharide band derived from sCR1-S/F was $BiNA_2F_2$. The analyses we performed do not establish linkages between the terminal and penultimate sugars that define sLe^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) versus sLe^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-). However, two factors make it likely that the glycans of sCR1-S/F do, in fact, terminate in sLe^x. First, it is known that in CHO cells, N-linked glycans are most commonly formed by β4GalT-1, and hence have the type-2 structure, Galβ1-4GlcNAcβ1- (Lee *et al.*, 2001). Second, the acceptor specificity of FT-VI is known to be restricted to type 2 chains (Costache *et al.*, 1997; Weston *et al.*, 1992).

During optimization of the sialylation reaction, we noted that incubation of sCR1 with either a low concentration of ST3Gal-III (10 mU/ml) for 24 h or a higher concentration (75 mU/ml) for 1 h produced a nearly maximally sialylated product. Even after incubation at the highest concentration of sialyltransferase tested (600 mU/ml for 24 h), a small fraction of monosialylated biantennary species persisted, perhaps due to steric hindrance at particular sites. Improved pharmacokinetics observed for the fully sialylated sCR1-S molecule as compared with sCR1 is probably a consequence of the added sialic acid blocking the interaction of terminal galactosyl residues with hepatic asialoglycoprotein receptors (Stockert, 1995).

We observed that FT-VI at 25 mU/ml fucosylates most sialylated biantennary glycans within 24 h. No significant differences were observed in catalytic activities of FT-VI expressed in the NSO cell line versus *Aspergillus niger* expression systems. The sCR1 polypeptide was shown to be stable following prolonged incubation with enzyme from either source.

In vitro glycosylation of sCR1 at the 10-g scale was carried out at enzyme concentrations selected to ensure nearly complete reaction at each stage. Success with the single experiment reported is consistent with the ability to predict useful scaled-up reaction conditions over a range of at least 40-fold based on mass of starting substrate. Both the ST3Gal-III and FT-VI enzymes used to glycosylate 10 g sCR1 were produced in *A. niger*, an expression system widely used for the manufacture of industrial enzymes in ton quantities. Although further scale-up would require refinement of incubation conditions, it can be estimated from present results that glycosylation of 1 kg of sCR1 might require 40,000 U ST3Gal-III and 20,000 U FT-VI, amounts that seem plausible to produce at reasonable cost in an industrial setting. To our knowledge, this is the largest scale reported enzymatic glycosylation of a glycoprotein to date by several orders of magnitude (Fischer and Dorner, 1998; Nemansky *et al.*, 1995; Paulson *et al.*, 1977; Raju *et al.*, 2001; Thotakura *et al.*, 1994).

The optimized conditions chosen for scale-up were very similar to the conditions used to generate material used for *in vivo* and *in vitro* studies. Compared with sCR1-sLe^x, sCR1-S/F was shown to have twice the number of sLe^x moieties and about a 10-fold higher apparent affinity for binding to E-selectin. This higher affinity presumably results from increased cooperativity in a multivalent binding reaction wherein sLe^x moieties distributed widely over sCR1-S/F engage multiple immobilized E-selectin molecules. In certain clinical situations, the anticomplement inhibitory and anti-inflammatory activity of sCR1-S/F could be effectively targeted via a similar mechanism to sites of inflammation where endothelial cells have been activated and have up-regulated expression of adhesion molecules including P- and E-selectin. sCR1-sLe^x has been shown to be superior to sCR1 in a complement- and selectin-dependent lung injury model (Mulligan *et al.*, 1999), a murine model of ischemic stroke (Huang *et al.*, 1999), moderating skeletal muscle reperfusion injury (Kyriakides *et al.*, 2001a), moderation of acid aspiration injury (Kyriakides *et al.*, 2001b), reducing ischemia/reperfusion injury in rat lung grafts (Schmid *et al.*, 2001), and a myocardial ischemia and reperfusion model in the rat. sCR1-sLe^x significantly reduced myocardial infarct size and was significantly more effective than sCR1 in reducing neutrophil infiltration into the infarction (Zacharowski *et al.*, 1999). It will be interesting to investigate whether sCR1-S/F is even more effective than sCR1-sLe^x in similar animal models.

Materials and methods

Complement proteins, antibodies, enzymes, and other reagents

Purified sCR1 and sCR1-sLe^x were prepared as previously described (Rittershaus *et al.*, 1999). Nucleotide sugars (CMP–sialic acid and GDP-fucose) were manufactured at Neose (Horsham, PA). CMP–sialic acid was prepared from CTP and sialic acid with recombinant CMP NeuAc synthetase (Shames *et al.*, 1991). GDP-fucose was either made from GDP-mannose using GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase/reductase, or purchased from Yamasa (Chiba, Japan). A gene encoding for a truncated, soluble form of ST3Gal-III (rat) was expressed in *A. niger* var. *awamori* dgr246 P2 using a variant of the expression vector pSL 1180 (Ward and Power, 2003). A 30–60% ammonium sulfate pellet was dissolved in 100 mM NaCl, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with 1 M NaCl, 20 mM MES, pH 6. rFT-VI (human) was expressed either in NSO cells or in *A. niger* as described as a soluble protein lacking the transmembrane domain. For the *A. niger* expressed protein, a 30-60% ammonium sulfate pellet was dissolved in 20 mM MES, pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM MES, pH 6. Soluble recombinant E-selectin was purchased from R&D Systems (Minneapolis, MN). Streptavidin–horseradish peroxidase conjugate (SA-HRP) was from Pierce (Rockford, IL), and biotinylated polyacrylamide polymer (PAA-sLe^x) was from GlycoTech (Rockville, MD). Anti-sCR1 monoclonal antibodies 6B1.H12 and 4D6.1 were prepared as previous described (Nickells *et al.*, 1998). Standards and glycosidases used in FACE analyses were from Glyko (Novato, California).

Preparation of sCR1-S

Lyophilized sCR1 (250 mg) was reconstituted and buffer exchanged into 50 mM Tris, 0.15 M NaCl, 0.05% NaN_3,

pH 7.2, using gel filtration columns (PD-10, Amersham Biosciences), and the concentration of sCR1 was adjusted to 5 mg/ml with the same buffer. Following addition of ST3Gal-III (150 mU/ml) and CMP-sialic acid (7 mM) the mixture was incubated at 32°C. A separate aliquot of the reaction mixture to which a trace amount of CMP-[^{14}C]sialic acid was added was incubated in parallel. From this, aliquot samples were withdrawn at various times and fractionated by isocratic HPLC/size-exclusion chromatography at 0.5 ml/min in 45% MeOH, 0.1% trifluoracetic acid (7.8 mm × 30 cm TSKG2000$_{SWXL}$ column, particle size 5 μm, TosoHaas). Incorporation of sialic acid into glycoprotein was calculated from the fraction of counts in the first eluted peak and the known concentration of sugar nucleotide.

Preparation of sCR1-S/F

After the sialylation reaction had proceeded for 48 h, GDP-fucose was added to a final concentration of 7 mM, $MnCl_2$ to 5 mM, and rFT-VI to 0.1 U/ml. A trace amount of GDP-[^{14}C]fucose was added to a separate aliquot, and both reaction mixtures were incubated at 32°C. Chromatography of the radiolabeled mixture as described showed the transfer of ~44 moles/mole sCR1-S after 48 h and 47 moles after 48 h. The product was provisionally designated sCR1-S/F.

Removal of nucleotide sugars and residual glycosyltransferases using ceramic hydroxyapatite and Q Sepharose chromatography

Glycosyltransferases and nucleotide sugars were removed from remodeled sCR1-S and sCR1-S/F by chromatography on ceramic hydroxyapatite (type I; BioRad, Hercules, CA) followed by Q Sepharose (Amersham Biosciences). Purity was assessed by RP-HPLC on a Poros R1/10 column (4.6 mmD/100 mmL, Applied Biosystems, Framingham, MA).

Optimization of sialylation and fucosylation reactions prior to scale-up

sCR1 was thawed slowly at 4°C and buffer exchanged into 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, using a PD10 column. *In vitro* sialylation of sCR1 (5 mg/ml) was evaluated using varying amounts of ST3Gal-III, 5 mM CMP-sialic acid, in the presence of 0.02% sodium azide at 32°C for 24 h. A trace amount of CMP-[^{14}C]sialic acid was added to an aliquot to monitor incorporation of radioactive sialic acid as described.

To the product (sCR1-S) of the reaction performed at a sialyltransferase concentration of 100 mU/ml (still containing the sialylation reagents) was added $MnCl_2$ and GDP-fucose, each to a final concentration of 5 mM, varing amounts of FT-VI, and a trace amount of GDP-[^{3}H]fucose. The resulting reaction mixture was incubated at 32°C for 24 h. Incorporation of radioactive fucose into the product (sCR1-S/F) was monitored as described for sialic acid.

sCR1 remodeling at 10-g scale

Purified sCR1 (10 g) was dialyzed exhaustively at 4°C against 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, adjusted to a concentration of 5 mg/ml with the same buffer, and incubated with ST3Gal-III (200 mU/ml) and CMP-sialic acid (5 mM) for 36 h at 32°C in a final volume of 2 L. After 36 h, an aliquot containing the sialylated product (sCR1-S) was withdrawn for analysis and the following reagents (final concentrations) were added: rFT-VI (100 mU/ml), GDP-fucose (5 mM), $MnCl_2$, (5 mM). After further incubation at 32°C for 36 h, a precipitate (manganese phosphate) was removed by centrifugation at 3000 × *g* for 5 min, and the sialylated and fucosylated product (sCR1-S/F) was stored at −70°C.

Monosaccharide analysis by HPLC

The neutral and amino sugar composition of glycoproteins was determined after trifluoroacetic acid hydrolysis and reductive amination with anthranilic acid by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1994). Sialic acid content was determined after sodium bisulfate hydrolysis and reaction with o-phenylenediamine by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1995).

Carbohydrate analysis by FACE

Carbohydrate sequencing and electrophoresis by FACE (Glyko and ProZyme, San Leandro, CA) was performed as previously described elsewhere (Picard *et al.*, 2000).

Carbohydrate analysis by 2-AA HPLC and MALDI-TOF MS

Glycans were released by PNGaseF and labeled with 2-AA according to the method described by Anumula and Dhume (1998) except that the labeled glycans were purified on cellulose cartridges (Glyko) according to the manufacturer's instructions. 2-AA-labeled N-glycans were analyzed using a Shodex Asahipak NH_2P-50 4D amino column (4.6 mm × 150 mm). The two solvents used for the separation were (A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and (B) 5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water. The column was eluted isocratically with 70% A for 2.5 min, followed by a linear gradient from 70% to 5% A over a period of 97.5 min, and a final isocratic elution with 5% A for 15 min. Eluted peaks were detected using fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 420 nm.

For MALDI-TOF analysis, a small aliquot of the 2-AA-labeled N-glycans was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 μm pore, 47 mm diameter) floating on water. The dialyzed aliquot was dried in a vacuum centrifuge, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water:acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Glycan structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully characterize isobaric structures.

SDS–PAGE

sCR1 samples before and after *in vitro* enzymatic remodeling were separated on 8–16% gradient Tris-glycine

polyacrylamide gels and stained with colloidal blue Coomassie stain. Gels, staining solutions, and molecular weight standards were obtained from Invitrogen (Carlsbad, CA).

Assays of complement regulatory activity

The inhibition of complement-mediated lysis of antibody-sensitized sheep erythrocytes (classical pathway) was assessed as previously described (Scesney *et al.*, 1996).

E-selectin binding assay

E-selectin binding assays were performed according to previously reported methods (Weitz-Schmidt *et al.*, 1996). Flat-bottom 96-well microtiter plates were coated with 5 μg/ml recombinant human E-selectin (R&D Systems) in 150 mM NaCl, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4 (HEPES-buffered saline, HBS). Coated wells were blocked with 2% bovine serum albumin/HBS. Varying concentrations of sCR1 or sCR1-sLe^x were added to the plate. A complex of a biotinylated polyacrylamide polymer containing sLe^x (PAA-sLe^x, GlycoTech) and SA-HRP was prepared. A dilution of this conjugate complex was added to the wells containing sCR1 or sCR1-sLe^x or buffer and incubated for 90 min at room temperature. The wells were washed with HBS/$CaCl_2$ and 3,3',5,5'-tetramethylbenzidine substrate (KPL) was added to each well. Color was allowed to develop for 15 min, and the reaction was stopped with 2.0 N H_2SO_4. Bound PAA-sLe^x complex was measured by determining the absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Pharmacokinetic analysis in rats

Male Sprague-Dawley rats (~250 g), with in-dwelling jugular vein cannulas were purchased from Taconic (Germantown, NY) or Harlan Sprague Dawley (Indianapolis, IN). The catheters were periodically flushed with 0.9% saline followed by either heparinized glycerol (1:4 glycerol/333 IU heparin/ml) or heparinized saline (333 IU/ml) to ensure patency.

Animals were injected with sCR1 or sCR1-S (10 mg/kg) via the lateral tail vein as a bolus at time 0. Blood samples were obtained at timed intervals from the jugular vein cannula. The levels of sCR1 and sCR1-S present in the plasma samples were measured by a previously described enzyme-linked immunosorbent assay (Rittershaus *et al.*, 1999). Briefly, microtiter plates were coated with anti-sCR1 monoclonal antibody 6B1.H12 and captured sCR1 from a sample was detected with an HRP-conjugated anti-sCR1 monoclonal antibody 4D6.1. Pharmacokinetic data was analyzed using WinNonlin (Pharsight, Mountain View, CA).

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Abbreviations

2-AA, 2-anthranilic acid; AUC, area under the curve; CHO, Chinese hamster ovary; FACE, fluorophore-assisted carbohydrate electrophoresis; HBS, HEPES-buffered saline; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; RP-HPLC, reversed phase high-pressure liquid chromatography; SA-HRP, streptavidin–horseradish peroxidase; sCR1, soluble recombinant complement receptor type 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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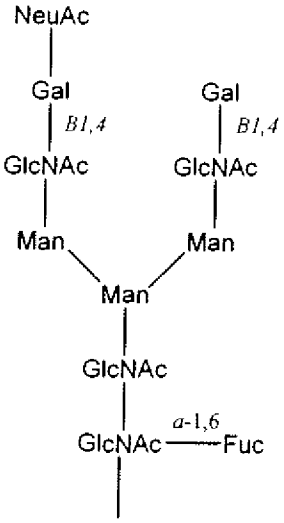
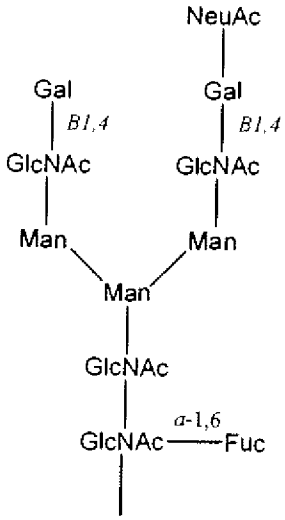
Exhibit 2C

N-linked glycan structures

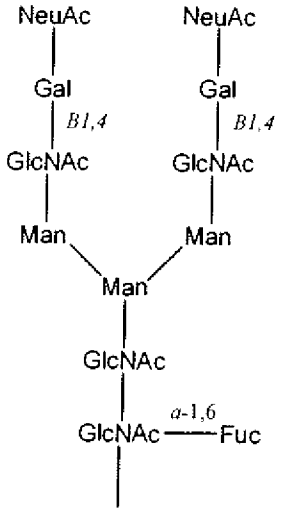
A.



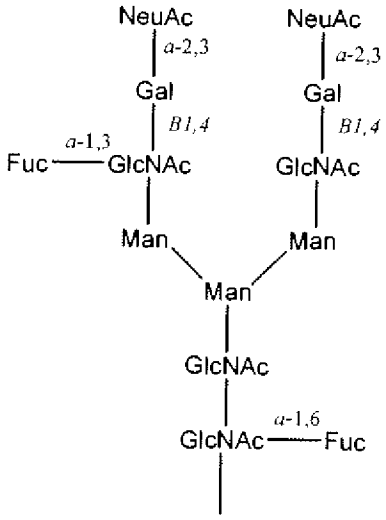


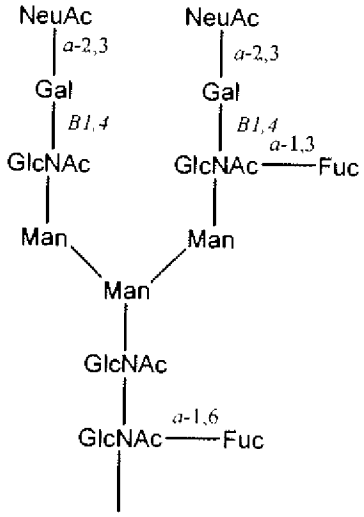
or

B.



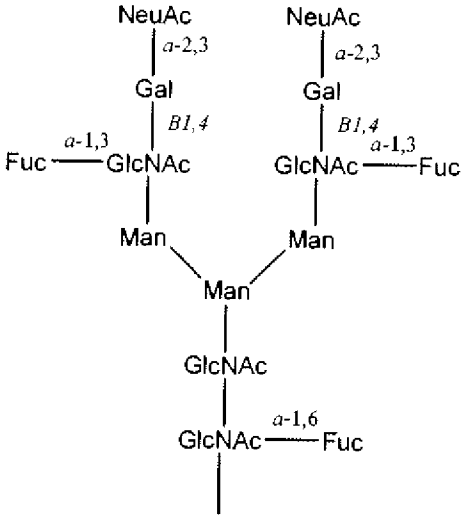
C.





or

D.



A. Monosialylated, biantennary core-fucosylated glycan

B. Disialylated, biantennary core-fucosylated glycan

C. Monofucosylated, disialylated, biantennary core-fucosylated glycan

D. Difucosylated, disialylated, biantennary core-fucosylated glycan

Exhibit 2D

Fucosylation Conditions

14:1 Donor : Acceptor ***Example 1,'806 Application***	***7:1 Donor : Acceptor*** ***Thomas***
2.5 mg/mL sCR1-S	5 mg/mL sCR1-S
5 mM GDP-fucose	5 mM GDP-fucose
0.05 U/mL FT-VI	0.1 U/mL FT-VI
32 °C for 2 days	32 °C for 36 hours

EXHIBIT V

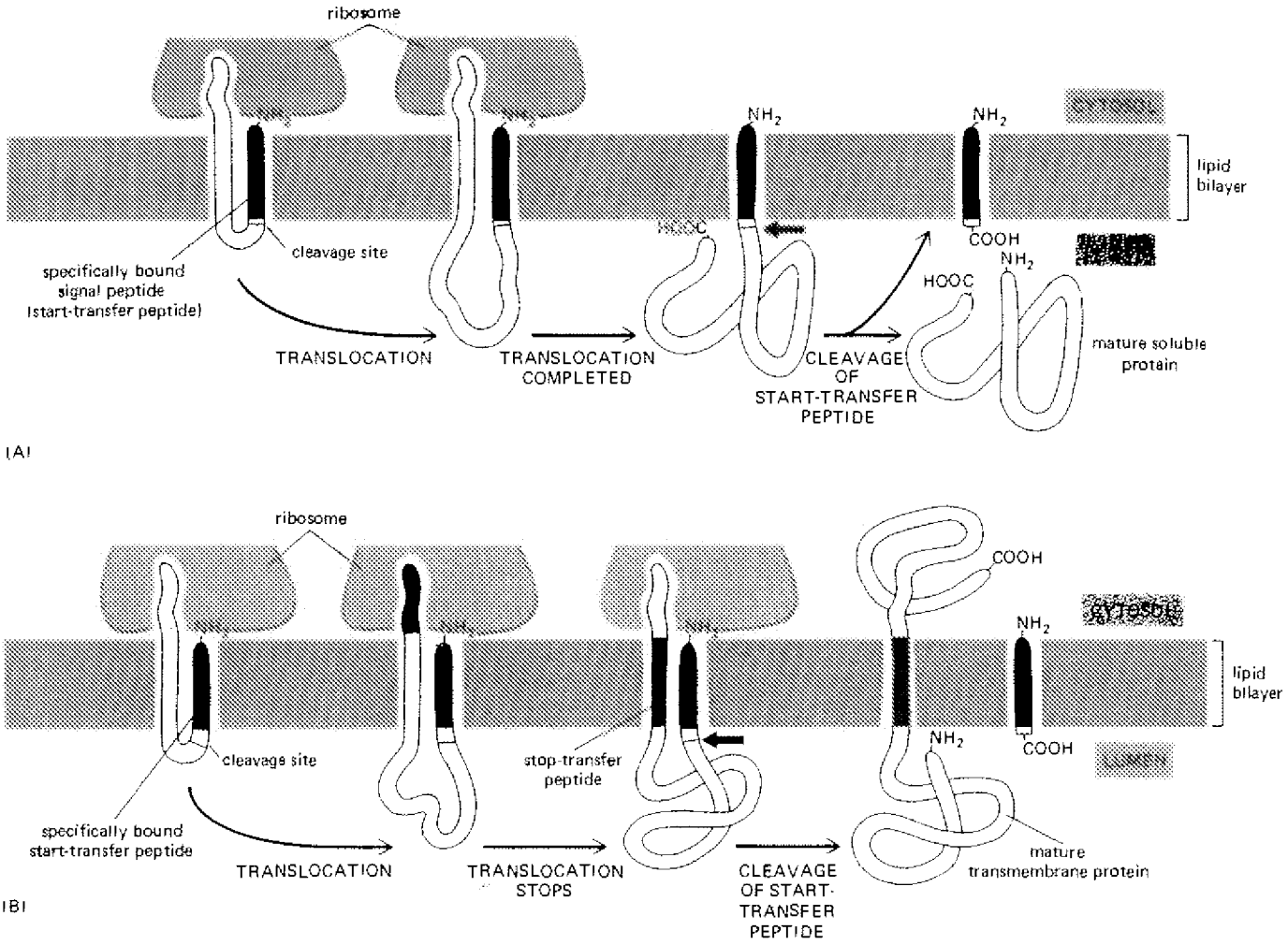


Figure 8–45 The topology of protein translocation across the ER membrane is illustrated for two simple cases. The translocational intermediate is thought to contain a loop of polypeptide chain, in which the signal peptide (also called the start-transfer peptide) forms half of the stem of the loop and the region of the polypeptide being translocated across the membrane at any instant forms the other half. When there is one start-transfer peptide and no stop-transfer peptide, the entire polypeptide is translocated across the membrane, and cleavage of the start-transfer peptide releases the mature protein into the lumen of the ER as a soluble protein (A). When one start-transfer peptide and one stop-transfer peptide are present, translocation stops when the stop peptide enters the stem of the loop while protein synthesis continues on the cytosolic side of the membrane; after cleavage of the start-transfer peptide, the mature protein is left spanning the lipid bilayer of the ER with one domain protruding on each side (B).

p. 293). It is thought that an internal signal peptide serves as a start-transfer signal in these proteins and initiates translocation, with each translocation event proceeding until the next stop-transfer peptide is reached. Thus the fundamental unit translocated is a loop of polypeptide between two hydrophobic segments (one start-transfer peptide and one stop-transfer peptide), with both of these peptides serving as α-helical membrane-spanning domains in the mature protein. A possible mechanism that might insert one such loop into the membrane is illustrated in Figure 8–46. In complex multipass transmembrane proteins, in which many hydrophobic α helices span the bilayer, a second start-transfer peptide would reinitiate translocation farther down the chain until the next stop-transfer peptide halted it once again, and so on for subsequent start-transfer and stop-transfer peptides (see Figure 8–48D).

8.2.8 The General Conformation of a Transmembrane Protein Can Often Be Predicted from the Distribution of Its Hydrophobic Amino Acids[40]

Although stop-transfer peptides are generally more hydrophobic than start-transfer peptides, they can sometimes act as start-transfer peptides if their location in a protein is changed. Thus the distinction between hydrophobic start-transfer and stop-transfer peptides results in part from the order in which they occur in the nascent polypeptide chain. It seems that the translocation machinery in the ER membrane begins scanning an unfolded polypeptide chain for hydrophobic segments at its amino terminus and proceeds toward the carboxyl terminus—in the same direction as the protein is synthesized. The SRP recognizes the first appropriate segment and thereby sets the "reading frame." The next appropriate hy-

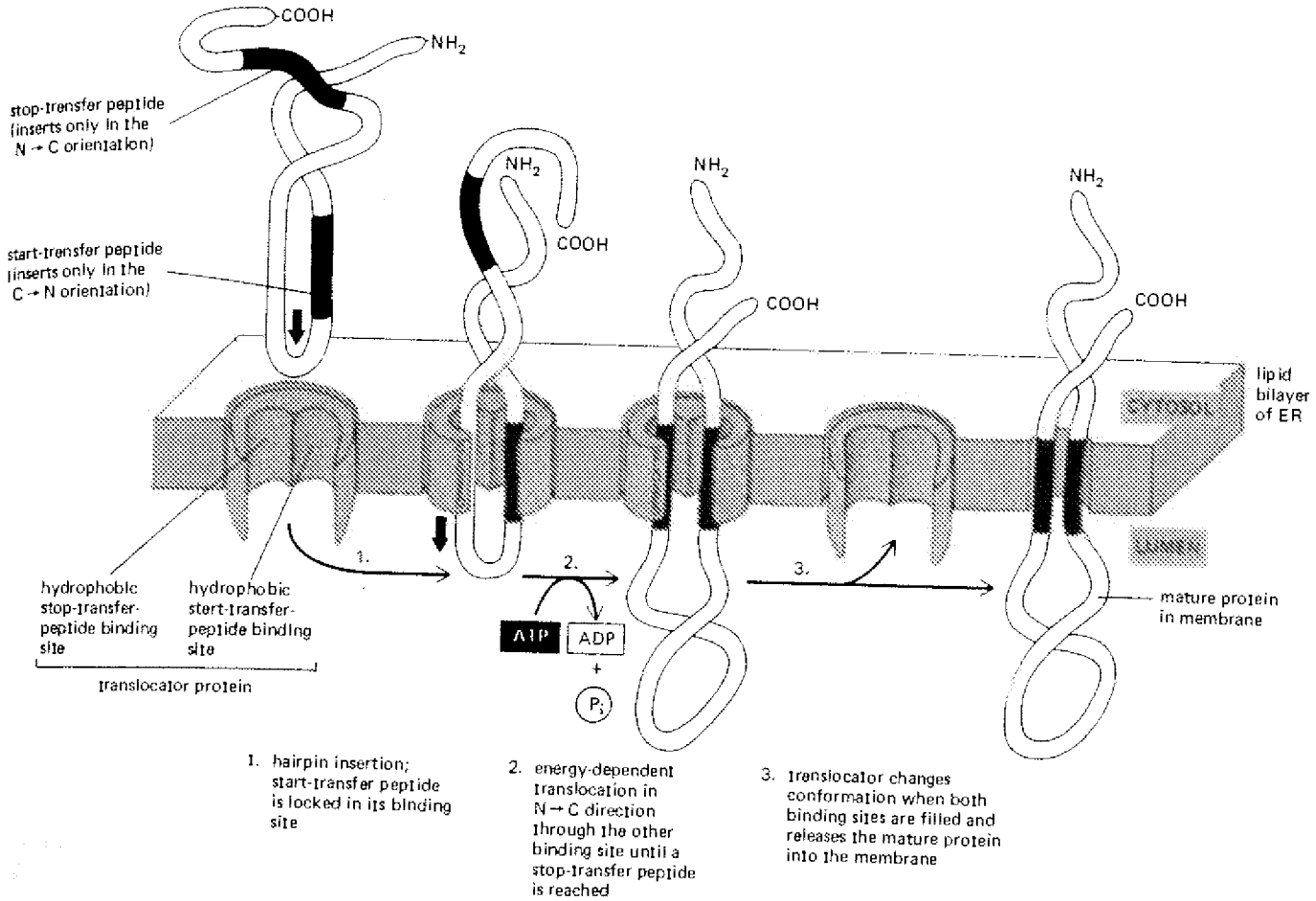


Figure 8–46 A hypothetical model for the insertion of an internal loop of polypeptide chain into the lipid bilayer of the ER. A protein translocator is postulated to have two conformations, "closed" and "open." On binding the start-transfer peptide of a protein to be translocated, the translocator enters the closed state and becomes active in translocation. But it flips back to an inactive, open conformation and discharges its protein as soon as a stop-transfer peptide enters its other binding site.

hydrophobic segment is recognized as a stop-transfer peptide, causing the region of the polypeptide chain in between to be threaded through the membrane (see Figure 8–46). It has been postulated that a similar process continues until all of the hydrophobic regions in the protein are inserted into the membrane.

This mechanism for membrane insertion means that one can often predict the topography of a membrane protein from its amino acid sequence. One begins by scanning for contiguous regions of about 20–30 amino acid residues with a high degree of hydrophobicity. These segments are long enough to span a membrane as an α helix, and they can often be identified by means of a *hydropathy plot* (Figure 8–47). The topology of the polypeptide chain can then be predicted on the assumption that the first segment, scanning from the amino terminus, has a start-transfer function and that stop-transfer peptides thereafter alternate with further start-transfer peptides. Four examples are illustrated in Figure 8–48.

Because membrane proteins are always inserted from the cytosolic side of the ER in this programmed manner, all copies of the same polypeptide chain will have the same overall orientation in the bilayer. This generates an asymmetrical ER membrane in which the protein domains exposed on one side are different from those domains exposed on the other. This asymmetry is maintained during the many membrane budding and fusion events that transport the proteins made in the ER to other cell membranes (see Figure 8–10), and it therefore determines the orientation of the proteins in these membranes as well.

When proteins are dissociated from a membrane and reconstituted into artificial lipid vesicles (see p. 287), a random mixture of right-side-out and inside-out orientations usually results. Thus the protein asymmetry observed in membranes is thought to result solely from the process by which proteins are inserted into the ER membrane from the cytosol.

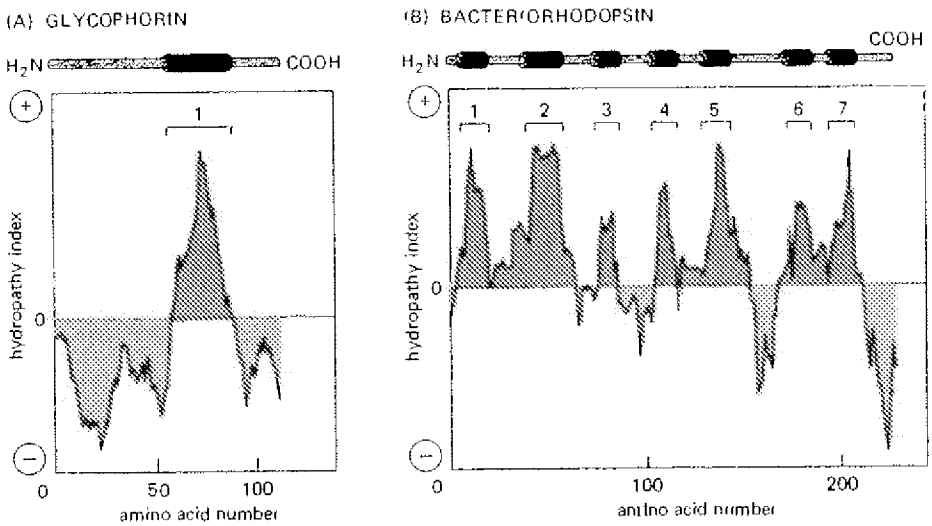


Figure 8–47 Localization of potential hydrophobic membrane-spanning segments in a polypeptide chain through the use of hydropathy plots. The free energy needed to transfer successive segments of a polypeptide chain from a nonpolar solvent to water is calculated from the amino acid composition of each segment using data on model compounds. This calculation is made for segments of a fixed size (usually around 10 amino acid residues), beginning with each successive amino acid in the chain. The "hydropathy index" of the segment is plotted on the *Y* axis as a function of its location in the chain. A positive value indicates that free energy is required for transfer to water (that is, the segment is hydrophobic), and the value assigned is an index of the amount of energy needed. Peaks in the hydropathy index appear at the positions of hydrophobic segments in the amino acid sequence. Two examples are shown: (A) glycophorin (see p. 291) has a single membrane-spanning hydrophobic domain and one corresponding peak in the hydropathy plot; (B) bacteriorhodopsin (see p. 293) has seven membrane-spanning helices and seven corresponding peaks in the hydropathy plot. (Adapted from D. Eisenberg, *Annu. Rev. Biochem.* 53:595–624, 1984.)

Figure 8–48 The topology of a membrane protein is dictated by alternating stop-transfer and signal (start-transfer) peptides. In all the examples shown, signal peptides are not cleaved. The hypothetical protein translocator is assumed to function in the manner illustrated previously in Figure 8–46. (A) When an amino-terminal signal peptide is not cleaved and no stop-transfer peptide is present, a membrane protein with a single carboxyl-terminal domain that protrudes on the luminal side of the ER membrane is generated. (B) When the signal peptide is internal, a membrane protein with an amino-terminal cytoplasmic domain and a carboxyl-terminal luminal domain is produced. (C) When a stop-transfer peptide follows an internal signal peptide, three separate domains will protrude from a membrane protein. (D) Membrane proteins that span the bilayer many times can be generated by a simple extension of the same principles, employing alternating signal peptides and stop-transfer peptides.

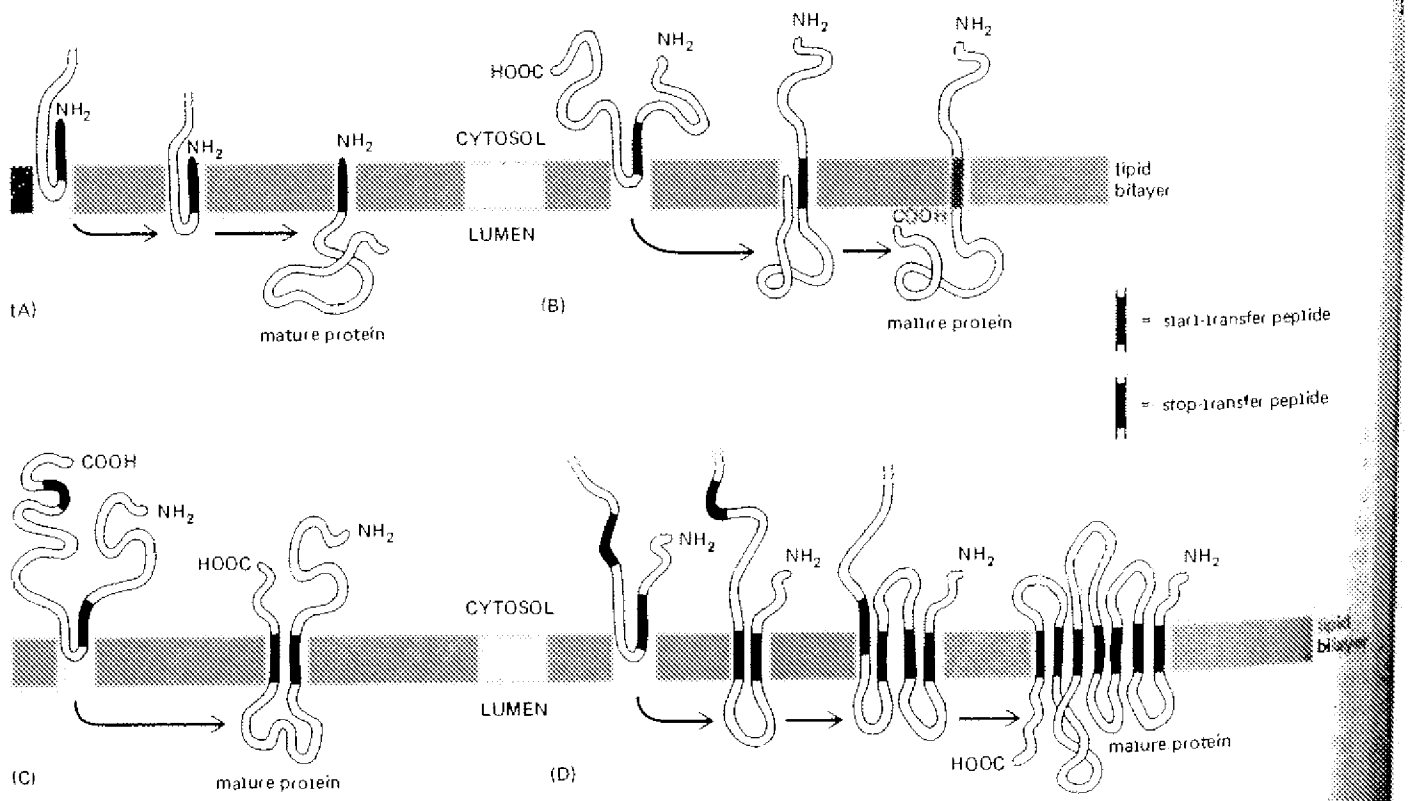


EXHIBIT VI

Under 35 U.S.C. § 112, First Paragraph, written description

Claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-55, 65-68, 70-77, and 79-86 are rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matters not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicants respectfully submit that the claimed *in vitro* methods are directed to the use of a group of well known enzymes, *i.e.*, fucosyltransferases, which are adequately described in the specification. In particular, on page 26, lines 1-29, the specification describes various fucosyltransferases useful for the present invention including references that contain the sequence information of these fucosyltransferases. It has also been acknowledged by the Office Action that the sequence information of fucosyltransferases are well known and publicly available. In addition, information is readily available to one skilled in the art with respect to conserved amino acids, binding sites for GDP-fucose, catalytic domain, etc. in the family of fucosyltransferases (See also Exhibit C as discussed above). Therefore, by identifying the enzyme, *i.e.*, fucosyltransferase and by providing structural descriptions, *e.g.*, lacking membrane anchoring domain, the specification clearly describes the fucosyltransferases useful for the claimed methods.

It has long been held by the court that what is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. See MPEP 2163 IIA 3(a) and Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d at 1384, 231 USPQ at 94. If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate written description requirement is met. See, *e.g.*, Vas-Cath, 935 F.2d at 1563, 19 USPQ2d at 1116.

Applicants respectfully submit that, in addition to the detailed descriptions provided by the specification, the examples provided by the specification further demonstrate that the inventors of the present invention were in possession of the claimed invention at the time of filing. Specifically Example 1 describes the use of FucT-VI *in vitro* according to the methods

provided by the present invention to achieve substantially uniform glycosylation pattern for glycopeptides. In Example 2, the inventors demonstrated that while both FucT-VI and FucT-V could be used for *in vitro* glycosylation according to the methods taught by the present invention, FucT-VI was capable of incorporating approximately 8-fold more fucose than FucT-V. Example 3 demonstrate that fucosylation by FucT-VI *in vitro* can be proceed by a sialylation step as taught by the present invention. Therefore, the examples provided by the specification at the time of filing clearly show that the inventors were in possession of the *in vitro* methods, *e.g.*, using fucosyltransferases *in vitro* to achieve substantially uniform glycosylation patterns for glycopeptides.

Furthermore, Applicants respectfully submit that, contrary to the Office Action's assertion that "many structurally unrelated polypeptides are encompassed within the scope of these claims", members of fucosyltransferases used in the present invention are closely related in terms of their structure and function and the specification discloses more than one species of fucosyltransferases useful for the *in vitro* methods. For example, it is known in the art that members of fucosyltransferases share a very high degree of sequence homology with respect to their functional domains. The specification describes more than ten (10) individual members of fucosyltransferase family suitable to be used for the present invention. See page 26, lines 1-29 in the specification. In addition, the examples provided in the specification demonstrate the use of at least two individual fucosyltransferases in the *in vitro* method provided by the present invention. Therefore, the descriptions provided by the specification fully support the scope of the claimed methods.

In summary, the *in vitro* methods using fucosyltransferases for substantially uniform glycosylation is fully described and supported by the specification. Withdrawn of the rejection is respectfully requested.

Under 35 U.S.C. § 103

Claims 1-4, 6, 8, 10-17, 19-21, 65-68, 70-77, and 83-86 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Seed, *et al.*, (PCT Publication No. 96/40881) ("Seed"), or